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Original Article

Comparative Evaluation of Remineralization Potential and pH Change of GC Tooth Mousse Plus and Alcoholic Extract of Cocoa Powder, and Antibacterial Efficacy against *Streptococcus mutans*: An *In Vitro* Study

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Abstract

Context: Dental caries in its early stages is a reversible phenomenon for which various remineralizing agents have been used, most common being fluoride, casein phosphopeptide-amorphous calcium phosphate. Plant extracts have also been researched for their remineralizing properties, as alternative to fluoride containing agents.

Objectives: The aim of this study is to evaluate and compare the remineralization potential, pH change, and effect on *Streptococcus mutans*, of a fluoride remineralizing agent and alcoholic extract of cocoa powder, in an *in vitro* setup.

Methods: Eighteen extracted premolars were prepared and tested for surface microhardness at three intervals: Baseline, demineralization, and remineralization. Demineralization was carried out using demineralizing solution until white spot lesion was seen, following this remineralization was carried out with Group A (GC Tooth Mousse Plus) and Group B (alcoholic extracts of cocoa powder). pH change: Dilutions of both solutions were checked for pH changes from initial, 5, 10, and 15 min intervals. Antimicrobial activity: Zones of inhibition at different concentration of solutions of both groups were checked.

Results: Statistically significant difference was observed in the values from demineralization to remineralization in both groups. Statistical analysis was performed using SPSS Version 19 software. Comparison of hardness, pH, and antimicrobial activity were done by independent t-test.

Conclusion: Both GC Tooth Mousse Plus and alcoholic extract of cocoa powder are equally effective as remineralizing agents, GC Tooth Mousse Plus is seen to be slightly more effective.

Key words: Remineralization, Casein phosphopeptide-amorphous calcium phosphate, Cocoa extract, GC Tooth Mousse Plus

INTRODUCTION

Dental caries, the most common oral health-related issue, is a multifactorial disease and has high global prevalence and incidence rates. Enamel is under constant influences of oral milieu, salivary fluctuations, and pH changes by accumulated food debris resulting in bacteria and their products. Caries is not a continuous and unidirectional process, rather a cyclic event consisting of periods of remineralization and demineralization, predominance of latter leads to a subsurface lesion, then cavitation.

The diagnosis and treatment of dental caries plays a very important role in maintaining oral health, for which research is never ending. The process of tackling dental caries had evolved from various treatment approaches

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aimed at restoring the tooth to a sound structure and function, to, development of novel and efficient diagnostic aids that enable detection at earlier stages, encouraging the concept of minimally invasive dentistry that is advocated, in the recent times. Remineralizing agents are non-invasive approaches to deal with the early carious lesions and are significant in decreasing their progress and clinical management of the disease.

Various remineralizing agents have been used in dentistry, fluoride being the most widely accepted, used, and incorporated in both in-office and home applications. Apart from fluoride used in varnish, gels, toothpastes, and tablets, other agents that have been developed over the years are casein phosphopeptide (CPP), amorphous calcium phosphate (ACP), bioactive glass, tricalcium phosphate, xylitol, CPP-ACP, and casein phosphopeptide-ACP fluoride (CPP-ACPF) have been used. Fluoride which has been in use since a long time basically forms a complex called fluorapatite replacing lost ions from the enamel, making it more resistant to dissolution by bacterial products and promoting remineralization. The anti-caries action of topical fluorides is now universally accepted and has been the subject of several reviews and meta-analyses of clinical data.[1]

CPP contains the amino acid cluster sequence of -Ser(P)-Ser(P)-Ser(P)-Glu-Glu can be purified as CPP-ACP nanocomplexes by selective precipitation, ion exchange.[2] These sequences are reservoir of calcium and phosphate ions incorporated in GC tooth mousse by Recaldent™, which have a synergistic action with fluoride (900 ppm) as in the GC Tooth Mousse Plus.

However, the use of fluoride for caries prevention is limited due its toxicity issues.[3] Hence, natural agents such as green tea,[4] grape extract,[5] cocoa bean husk, and cocoa extracts[6,7] have been researched.[8] Theobromine containing dentifrices claims to remineralize enamel lesions effectively.[9] Theobromine is a natural alkaloid derived from cacao beans (*Theobroma cacao* L.) containing secondary metabolites in the form of purine alkaloids derived from xanthine.[10] It is an alkaloid readily available in cocoa (240 mg/cup) in both cocoa bean and its husk. The mean theobromine content of cocoa beans is approximately 20.3 mg/g.[11]

Therefore, in the current study, fluoride in conjunction with CPP-ACP system, that is, CPP-ACPF is compared with alcoholic extract of cocoa containing theobromine, to assess the remineralization of enamel, antimicrobial action against *Streptococcus mutans,* and pH changes in an *in vitro* model.

MATERIALS AND METHODS

Materials

(1) Twenty intact premolars indicated for orthodontic extraction
(2) Thymol in distilled water for storage
(3) Demineralizing solution
(4) Alcoholic extract of cocoa powder
(5) Fluoride containing remineralizing agent (GC Tooth Mousse Plus)
(6) Artificial saliva
(7) Strains of *S. mutans*
(8) Agar plates for culturing
(9) Pocket pH meter.

Methodology

One kilogram of cocoa powder was treated in with 5 g of cellulose in 4.75 l of distilled water at 50°C for 4 h. Ethanol was then added up to 50 v/v of final concentration and mixture was refluxed for 1 h. After filtration, ethanol was removed by evaporation and aqueous solution lyophilized to form a powder. It is diluted with distilled water to attain a concentration of 0.05 mg/ml.

Evaluation of Surface Microhardness

Twenty premolars indicated for orthodontic extraction were obtained, only intact premolars, with the absence of cracks, caries, and defects were sorted and stored in thymol solution. The crowns were separated from the roots using a diamond disc and straight handpiece under water cooling. The crowns were mounted in circular resin blocks such that the buccal surface was exposed. The exposed tooth surface was polished slightly using a fine grit sandpaper to obtain a flat surface, for measuring surface microhardness. Samples were then stored in deionized water until further testing.

Baseline microhardness (SMH-1)

Specimens were thoroughly rinsed and dried. The exposed enamel surface was tested using a Diamond indenter of Microhardness Tester, Reichert Austria Make (Sr. No.363798), subjected to a load of 50 g. Two indentations were made on the surface at two different points, on each sample, to avoid discrepancy and their average was taken as the final value.

Demineralizing solution

Freshly prepared demineralizing solution with pH adjusted between 4.5 and 5.5 (critical pH) with contents as follows: Calcium 2.0 mMol/L, phosphate 2.0 mMol/L, and acetic acid 75.0 mMol/L was used for demineralization of surface enamel. All the specimens were immersed individually in the solution till white spot lesion was seen on the surface.

Second microhardness test (SMH-2)

It was conducted after white spot lesions were seen on exposed enamel surface, similar to that of baseline, for all 20 specimens.

Remineralization protocol

The samples were divided into two groups, Group A (10 samples) was immersed individually in alcoholic extract of cocoa powder and Group B (10 samples) was immersed in GC Tooth Mousse Plus, with equal dilutions with distilled water for both groups. This process was carried out every day 15 min for 7 days, in between the samples were stored in deionized water.

Third microhardness test (SMH-3)

It was carried out after the remineralization protocol using values of two indentations on the surface to obtain an average value for all the samples individually.

Antimicrobial Efficacy

To check the antimicrobial efficacy of both Group A (alcoholic extract of cocoa powder) and Group B (GC Tooth Mousse Plus) against *S. mutans*, organism was grown on MHA agar plates, and wells were created, in which three different concentrations 0.1. 0.5, and 1 ml were checked for zone of inhibition, for both groups.

pH Analysis

pH analysis was done using a pocket pH meter (Digital). Both the solutions were mixed with artificial saliva, and pH was checked initially, and then later at intervals of 5, 10, and 15 min. Three readings were taken and their average was calculated.

RESULTS

The values for baseline, demineralization, and remineralization for all 20 samples, average of two values, per sample are listed in Table A1.

On using concentrations of both products at 0.1 ml, 0.5 ml, and 1 ml for alcoholic extract of cocoa and 0.1 g, 0.5 g, and 1 g GC Tooth Mousse Plus, no inhibition of *S. mutans* is seen. This indicates that both the products do not affect the growth of *S. mutans in vitro* [Table A2].

The results of pH test and comparison of these values in Table A3, where only minor fluctuations are seen, show no significant *P* value at different intervals.

All the statistical analyses were performed using SPSS Version 19 software. Comparison of change in hardness and pH at each interval in each group was using repeated measure ANOVA test followed by *post hoc* test for pairwise comparison. Comparison of hardness and pH in between two groups at each interval was done using independent *t*-test. Comparison of antimicrobial activity in between two groups at each interval was done using independent *t*-test. Level of significance was kept at $P \leq 0.05$.

DISCUSSION

In this study, GC Tooth Mousse and alcoholic extract of cocoa powder containing theobromine have been tested for their remineralization properties. The enamel is made up of crystal lattice of hydroxyapatite predominantly calcium and phosphate ions, the concentrations of which influence the structural composition of sound and defective enamel. During phases that promote dissolution of enamel, ions are lost from this lattice, making it more susceptible to bacterial invasion. Remineralizing agents essentially restore the ions lost from the lattice creating a more stable structure that can withstand further breakdown promoting a temporary arrest of lesion progression, in its early stages of subsurface or white spot lesions.

Increase in the surface microhardness is seen on remineralization of carious enamel lesions Tencate *et al*., 1978, and Finke *et al*., 2000. Dental caries in its early stage of formation (non-cavitated) can be remineralized (Ten Cate and Featherstone, 1991; Featherstone, 2008). Fluoride, the gold standard in remineralization, is also preceded before use by caution of do not ingest. These risks of ingestion, especially in children, have made non-fluoride agents such as CPP-ACP and natural products among other systems as the subject of ongoing research.

Theobromine, a xanthine alkaloid found in extracts of cocoa powder and cocoa bean husk, has shown cariostatic effect at concentration of 71 times,[7] twice the protective effect on teeth as compared to fluoride at 142 times lesser concentrations as proposed by Carey.

In the present study, as seen in Graph A1, there is a significant difference seen, from demineralization to remineralization in both ($P > 0.5$), indicating that both agents are effective in remineralization of enamel lesions. However, when comparison of Group A and Group B was done, to see which agent was better as a remineralizing agent, [Graph A2] Group A – 5.91% as compared to Group B – 8.49%, Group A (alcoholic extracts of cocoa powder) has shown to be lesser effective in increasing surface microhardness as compared to Group B, but the difference is not enough to be statistically significant.

In this study, theobromine is used in calibrated concentrations to 1.1 mmol/l, which was the effective

concentration required to cause remineralization by Amaechi *et al*. The action of theobromine in cocoa extracts is owed to its ability to improve the surface chemical composition of enamel.[12,13]

Amaechi *et al*. have found that theobromine enhanced the resistance of remineralized surfaces to subsequent caries challenge. This action can be attributed to increased apatite crystallite size formed with theobromine (2 μm) as compared to 0.5 μm to that formed by fluoride crystals in an apatite forming medium. The results of this study are synchronous with studies conducted before by Frank Lippert,[14] Parvathy *et al*.,[9] Irawan *et al*.,[15] and Abdullah[16] where they have used theobromine incorporated within products and have concluded fluoride to be more effective. Irawan *et al*.[15] found that theobromine is not able to restore after demineralization on remineralization to its initial surface microhardness. Gundogar[17] and Kargul *et al*.[18] have found that the effect of theobromine is directly related to concentration used. In studies conducted by Duraisamy *et al*.[6] he has found theobromine to be more effective when compared to CPP ACPF and Nakumoto 2016[12] has seen better occlusion of dentinal tubules with a theobromine containing dentifrice as compared to fluoride containing dentifrice.

This may be attributed to two factors, that is, first, elemental theobromine is not used in this study, instead, theobromine is calibrated to an effective concentration in a naturally occurring plant product, and second, there are two synergistic systems involved in CPP- ACPF, mainly CPP and fluoride, which can also be a factor for the results being slightly in favor of GC Tooth Mousse Plus.

Since the values between both groups for remineralization are not statistically significant, it can be said that both GC Tooth Mousse Plus and alcoholic extracts of cocoa powder, both are effective in remineralization of tooth, as corroborated in studies conducted by Sadeghpour,[19] Amaechi *et al*.,[20] Kargul *et al*.,[18] Mahardhika *et al*.,[21] 2017, and Sulistianingsih *et al*.[22]

For the antimicrobial efficacy of GC Tooth Mousse Plus and alcoholic extracts of cocoa powder [Table A2], no significant results were seen in the study [Figure A1]. The main property of theobromine and GC Tooth Mousse Plus is enamel remineralization, evaluation of cariostatic property is an adjunct. Nevertheless, they have been shown to be effective by inhibiting biofilm formation on teeth and affecting the adherence properties of *S. mutans*, and other bacteria, in case of theobromine by inhibiting glucosyltransferases. This indirectly leads to inhibition or increase in time required for colonization of tooth surfaces that result in the display of inhibition of bacteria.[23]

In a study by Pinhero,[24] where CPP-ACP complex was added to GIC to check inhibition of *S. mutans*, there was inhibition seen immediately after placement, but counts increased after 6 months–1 year. This adds up to the previous observation where the adherence of bacteria is hampered due to the application of GC Tooth Mousse Plus.

Studies which have proven statistically significant activity with theobromine in terms of inhibition of bacteria[23] have used commercially available theobromine tooth paste, ingredients of which being Rennou™ (theobromine, calcium acetate, and sodium hydrogen phosphate) which can result in synergistic activity as compared to the use of elemental theobromine. Furthermore, a biofilm model may be more suited to evaluate this parameter, as its antimicrobial property is attributed to the prevention of biofilm formation.

As for pH activity, it is a very important factor and a predictor of dental caries. Decrease in pH below critical level causes the calcium ions to leach out of hydroxyapatite crystals and increase their concentration in saliva, which over prolonged periods of time causes demineralization and hollowing out of dentinal tubules creating pathways for bacteria to enter and cause dental caries. Prolonged lowered levels of pH cause irreversible demineralization, which then needs institution of treatment to restore it to normal form. Most remineralizing agents when mixed with saliva increase concentration of ions essential to form complexes with hydroxyapatite and cause rise in pH which helps in remineralization. In the present study, pH values were measured using a pH meter at 5, 10, and 15 min intervals, *in vitro* and no significant changes were seen. Both groups individually when mixed with artificial saliva had pH that was alkaline. The values did not fluctuate on standing, from 5, 10, and 15 min. The constant values were as a result of no external influencing factors on the solutions.

Shortcomings: Since it was an *in vitro* model, physiological conditions in the mouth could not be simulated, which could affect the study outcomes. Furthermore, naturally occurring theobromine devoid of any additives is seen.

CONCLUSION

Both the systems that were used in the present study were effective in remineralization, almost equally which can allow us to consider theobromine as an alternative to fluoride. Addition of which components to enhance remineralization properties of theobromine needs to be researched, since it is a very promising and safe agent as compared to fluoride. Relying on their antimicrobial activity is still doubtful, and more studies need to be done to test

this aspect. pH changes can also be tested effectively in an *in vivo* model. More research needs to be done to establish the currently available evidence.

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APPENDIX TABLES

Table A1: Baseline microhardness values for Group A and Group B, SMH-1 (microhardness in HV)

Surface microhardness 1 (baseline)						Surface microhardness 2 (demineralization)						Surface microhardness 3 (remineralization)					
Group A			Group B			Group A			Group B			Group A			Group B		
R1	R2	AVG	R1	R2	AVG	R1	R2	AVG	R1	R2	AVG	R1	R2	AVG	R1	R2	AVG
320	323	322	308	313	310	280	276	279	263	267	265	288	290	289	300	301	301
301	303	302	303	306	305	270	274	272	279	285	282	290	291	291	295	299	297
300	307	204	335	337	336	288	282	285	290	292	291	287	288	288	310	312	311
310	313	312	302	306	304	267	271	269	265	271	268	280	282	281	290	292	291
312	315	314	318	312	316	260	268	264	286	288	287	282	286	284	300	302	301
322	330	326	315	320	318	282	290	286	255	261	258	297	298	298	299	305	303
330	332	331	301	303	302	259	269	264	269	275	272	290	293	292	290	295	293
323	325	324	310	314	312	271	277	274	290	296	293	292	296	294	300	302	301
295	302	298	304	307	305	263	269	266	260	264	262	280	281	281	296	299	298
310	318	314	299	315	307	258	264	261	269	273	271	280	282	281	280	284	282

Table A2: Effect of Group A and Group B agents on *Streptococcus mutans*

Test organism	Product details	Result – zone of inhibition (mm)
Streptococcus mutans	0.1 ml extract	NI
Streptococcus mutans	0.5 ml extract	NI
Streptococcus mutans	1 ml extract	NI
Streptococcus mutans	0.1 g GC Tooth Mousse Plus	NI
Streptococcus mutans	0.5 g GC Tooth Mousse Plus	NI
Streptococcus mutans	1 g GC Tooth Mousse Plus	NI

NI: No inhibition. Since results showed no change, statistical analysis of this parameter could not be done

Table A3: Comparison of values of pH and their statistical analysis

Interval	Group A pH	Group B pH	Difference	*P* value
Initial	5.80±0.20	6.90±0.10	−1.10	0.001*
At 5 min	5.67±0.06	6.97±0.06	−1.30	0.001*
At 10 min	5.67±0.06	6.97±0.06	−1.30	0.001*
At 15 min	5.60±0.00[a]	7.00±0.00[a]	--	--

pH analysis of all specimens in Group A and B. Paired t-test; * indicates significant at $P \leq 0.05$

APPENDIX FIGURE

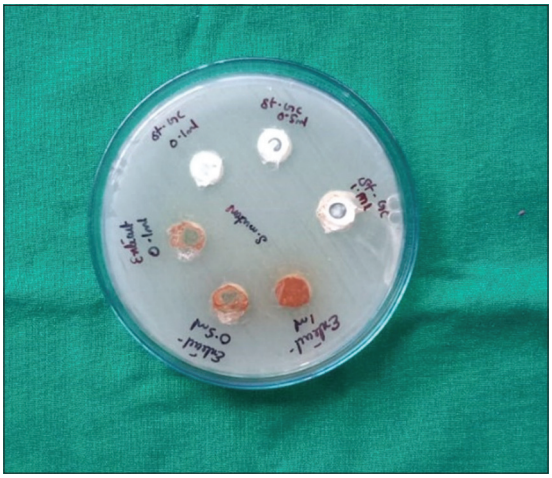
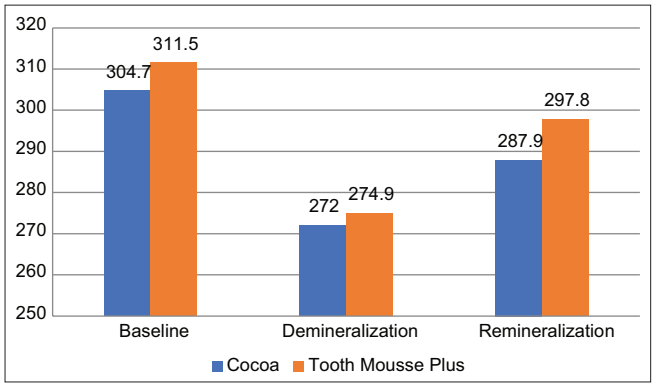
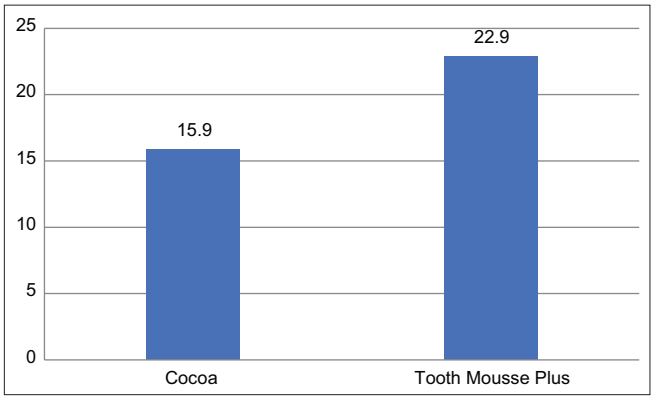


Figure A1: No zone of inhibition seen with both groups

APPENDIX GRAPH



Graph A1: Graphical representation of microhardness of Group A and Group B at different intervals. Significant difference seen from demineralization to remineralization in both groups



Graph A2: Increase in microhardness for both groups post-demineralization, difference is not statistically significant